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(54) Title: A METHOD FOR DETECTING DNA

(57) Abstract

The present invention relates to a method for detecting a target DNA in a sample which method comprises capturing a first DNA containing said target DNA from said sample, amplifying said target DNA using a polymerase chain reaction and then detecting said amplified target DNA.

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A METHOD FOR DETECTING DNA

The present invention relates generally to a rapid method for the detection of target DNA in a sample comprising capturing a first DNA containing a target DNA followed by amplifying target DNA by the polymerase chain reaction and then detecting the amplified target DNA.

The polymerase chain reaction (hereafter referred to as "PCR") using Taq polymerase or other thermostable DNA polymerases for amplifying specific DNA segments in vitro has had widespread applications including the detection of pathogens, the diagnosis of genetic disorders, forensic analysis and many genetic manipulations for research purposes[1,2]. Various sources of DNA have been used for PCR. Phage plaques or bacterial colonies carrying plasmid DNA have multiple copies of the DNA of interest and, hence, PCR can be performed directly without the need for DNA purification[3]. Some purification step is, however, generally required for PCR amplification of single copy genes of genomic DNA, especially if there is a mixed population of cells[4]. Such a circumstance occurs for blood borne infections (eg malaria) where only a small proportion of the blood cells harbour the pathogen. The purification procedures generally used (eg. making a pellet followed by proteinase K digestion) are relatively impractical for routine tests because they are complicated and require multiple manipulations. Many of the proposed diagnostic applications of the PCR are of the mass-screening type, such as screening donor blood for HIV 1, HIV 11, etc and for diagnosing carrier status in genetic diseases such as cystic fibrosis. Products are currently available to enable isolation of DNA from biological samples, but these products require several centrifugation steps to

obtain DNA ready for PCR amplification. Therefore, a practical, quick and simple technique of sample preparation/purification is required. The simple method of boiling whole cells and using clarified material for PCR has been successfully used for phage or plasmid DNA but has been unreliable with regard to whole blood, probably due to inhibitory substances such as haematin[4] in the large amount of cell debris. Even as little as 1 µl of whole blood in a 0.1 ml reaction can cause inhibition.

In work leading up to the present invention, a procedure was developed employing anti-histone antibodies to capture chromatin, and hence DNA, in a tube used for PCR. This strategy was devised to minimise the number of manipulations. The subject method is particularly useful for field studies and can be conveniently presented in kit form. This improvement to PCR extends also to the capture of DNA containing the target DNA sequence using, for example, DNA binding proteins. The use of antibodies to capture the first DNA is described herein as "immuno-PCR". The present invention, however, extends to both immunological and non-immunological means for capturing the first DNA.

Accordingly, one aspect of the present invention contemplates a method for detecting target DNA in a sample which method comprises capturing a first DNA containing said target DNA, amplifying said target DNA DNA using a PCR and then detecting said amplified target DNA.

In one preferred embodiment, the first DNA is captured using an antibody to an antigen associated with said first DNA such as an anti-histone antibody. In another preferred embodiment, the first DNA is captured using a DNA binding protein such as GCN4 or GST-GCN4.

As used herein "target DNA" refers to that region of said first DNA which is subject to amplification by the PCR and is determined by the appropriate primers used. The target DNA and first DNA may also be co-terminus, i.e. be the same molecule. Target DNA may comprise an entire gene or a portion thereof or may represent a particular region in, for example, prokaryotic, eukaryotic or proviral DNA. Although the immuno-PCR aspect of the subject invention is exemplified using anti-histone antibodies, its ambit extends to antibodies to any antigenic material, such as protein, that is tightly complexed to the first DNA. For example, the present invention extends to the use of antibodies directed to regulatory proteins capable of binding to specific (regulatory) regions of a first DNA. In one embodiment, the protein may bind to a first DNA in a particular organism from a mixture of organisms wherein the particular organism carries the specific regulatory protein associated with its genome. Accordingly, the present invention extends to the enrichment of DNA from specific cells (eg. cancer cells and pathogens and non-pathogens such as viruses, bacteria, parasites, mycoplasma, fungi and yeast) via antibodies to one or more antigenic molecules, eg. proteins, exclusive to said specific cells.

Target DNAs contemplated by the present invention include chromosomal and genomic DNA, proviral and other pathogenic DNA whether or not associated with chromosomal or genomic DNA of the host, oncogenic and normal and abnormal eukaryotic genes or DNA (such as those involved in cancers and genetic disorders).

In accordance with the present invention, the target DNA contained in said first DNA is isolated from a sample. By "sample" is meant to include a source of chromosomal or genomic DNA. For the performance of immuno-PCR, the

sample would include a source of histone-complexed or associated DNA or, as referred to above, antigenic material associated DNA, such as would be obtainable from eukaryotic cells and/or their extracts. A sample would include biological fluids, extracts, suspensions or samples, such as blood, lymph, respiratory fluid or extract, excreta, tissue, tissue-extracts and the like in animals, for example, mammals, humans, avian and reptilian species. The source may also be non-biological such as environmental and/or industrial effluent or solid waste. By "fluid" includes particular suspension as well as solution.

The antibodies used in the immuno-PCR contemplated herein may be polyclonal or monoclonal and naturally occurring or synthetic (eg. recombinant) and include parts of said antibodies. The source of such antibodies may be from mice, rat, goat, horse, rabbit, human or other animal; the choice dependent upon cost, ease of manipulation and quantity of antibody required. Techniques for generating polyclonal and monoclonal antibodies to histones are well known in the art.

Accordingly, in immuno-PCR, the target DNA is detected in a sample by capturing a first DNA containing said target DNA by contacting said first DNA with a binding effective amount of an antibody specific to an antigen associated with said first DNA for a time and under conditions sufficient for an antibody-first DNA complex to form, subjecting the captured first DNA to PCR to amplify said target DNA and then detecting said target DNA. Preferably, the antibodies are first immobilised to a solid surface. The solid surface could be glass or a polymer, for example cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene. The solid supports may be in the form of tubes, beads, discs

or microplates or any other surface for conducting immuno-PCR. Binding processes for immobilising antibodies to solid surfaces are well known in the art and include cross-linking, covalent binding or physically adsorbing the molecule to the insoluble carrier. The antibodies may also be immobilised by binding to a second set of antibodies specific to the first antibodies, said second set bound to the solid support. Furthermore, the capturing of the first DNA may occur after the formation of an antibody - first DNA complex by, for example, using antibodies specific to the first antibodies referred to above. According to this aspect of the present invention, a first DNA containing a target DNA is contacted with a binding effective amount of a first antibody specific to an antigen associated with said first DNA for a time and under conditions sufficient for a first antibody-first DNA complex to form, contacting said first antibody-first DNA complex to a antibody-binding effective amount of a second antibody, said second antibody immobilised to a solid support, amplifying the target DNA by PCR and then detecting said amplified target DNA. As with the first antibody, the second antibody may be monoclonal and/or polyclonal or parts or combinations thereof. In most preferred embodiments the antibody specific to the antigen associated with the first DNA is an anti-histone antibody and the antigen is histone.

Many variations in the binding and capturing of the first DNA exist but which do not fall beyond the scope of the present invention. All such variations are encompassed herein. Variants extend to the use of DNA binding proteins to capture a first DNA containing target DNA in place of anti-histone antibodies. Suitable DNA binding proteins include the double-stranded DNA binding specific protein - GCN4. The present invention therefore extends

to capturing and detecting target DNA in a sample using DNA binding proteins, amplifying said target DNA in said captured DNA using PCR and then detecting amplified target DNA. Other variations embodied within the scope of the present invention relate to the use of alternate cell capture methods. For example by capturing only those cells which contain the DNA, for example lymphocytes and removing potentially contaminating red blood cells prior to lysis the DNA can be prepared and submitted for amplification without precipitation and centrifugation. Yet another variation extends to the use of antibodies specific to the cells most likely to contain the target DNA. For example, to assist in the detection of the HIV genome, cells expressing the determinants CD4 and CD8 could be captured by coating tubes with specific antibodies to these markers.

In accordance with this aspect of the present invention a first antibody specific to a cell surface antigen of a cell or organism containing the target DNA is used to capture said cell or organism. Depending on the sample, unwanted or unbound cells or organisms may then be washed away. The cell is then lysed or otherwise disrupted to release a first DNA (e.g. genomic DNA) which contains a target DNA. The target DNA in the first DNA is then amplified using PCR and the amplified product detected. This method can be used to identify specific cell or organism types in a sample using antibodies to a cell surface antigen followed by amplification and detection of target DNA within the genomic DNA and/or as a rapid and convenient source of target DNA in a particular cell type in a sample.

Accordingly, the present invention extends to a method for the selective enrichment and/or isolation of target DNA and/or the identification of certain cell types in a

sample which method comprises contacting the cells in said sample with a first antibody specific to an antigen on a desired cell type for a time and under conditions to capture said cells, disrupting said cells to release a first DNA containing a target DNA, amplifying the target DNA therein and then detecting the amplified target DNA.

This method may also contain the additional step after the release of the first DNA of capturing same using either an antibody specific to an antigen associated with said first DNA (e.g. an anti-histone antibody) or using a DNA binding protein (e.g. GCN4 or GST-GCN4). In accordance with this embodiment, the capture of the first DNA may be by either a second antibody specific to an antigen associated with said first DNA or by using a DNA binding protein as discussed above. In a preferred embodiment, the first and second antibodies or the first antibody and the DNA binding protein are immobilised on a single solid support such as on the inside surface of a container adapted to receive and contain said sample. In another preferred embodiment, one or both antibodies are monoclonal antibodies.

Furthermore, in certain circumstances, the first DNA will be the same as the target DNA or the target DNA will reside in the same strand as a first DNA strand or may be on a different strand but still associated, eg. by hydrogen bonds, to the first DNA strand. The present invention further extends, therefore, to capturing and detecting target DNA directly or in addition to when the target DNA is associated in any form with said first DNA.

Any number of means exist for detecting the amplified target DNA. One convenient means is the use of the ELISA. Other examples include, spectrophotometric and radioimmunoassay procedures or the amplified DNA assay

(ADA) as disclosed in International Patent Application No. PCT/AU89/00526 and which is incorporated herein by reference. All such means are within the scope of the present invention.

The present invention is particularly applicable to the ADA. An example of the applicability of these two procedures is shown in Example 6 herein. This example shows the ease by which the improved PCR can be used to capture target DNA from human blood cells and to incorporate the DNA binding sequences for TyrR or GCN4 into amplified DNA as described for ADA.

Accordingly, another aspect of the present invention contemplates a method for detecting target DNA in a sample which method comprises capturing on a first solid substrate a first DNA containing said DNA in said sample using an antibody specific to an antigen associated with said target DNA (such as an anti-histone antibody) or a DNA binding protein and then detecting said target DNA by the ADA comprising incorporating a first ligand into said DNA by polymerase chain reaction using a set of primers wherein one of the primers bears the ligand and contacting the so treated DNA with a second solid substrate having a binding reagent for said ligand immobilised thereon and subjecting said captured amplified DNA to a detecting means or alternatively contacting said captured amplified DNA with a detection reagent which is capable of binding to a second ligand previously incorporated into said amplified DNA by the polymerase chain reaction using a set of primers wherein one of the primers bears the ligand capable of binding to a detection reagent.

The present invention also extends to a kit for detecting target DNA sequences which kit comprises in compartmental form a first container adapted to contain an immobilised antibody specific to an antigen associated with said first DNA (e.g. an anti-histone antibody) or a DNA binding protein; a second container adapted to contain a sample containing a source of a first DNA; a third container adapted to contain the reagents for a PCR; and a fourth container adapted to contain a detection means. One skilled in the art will immediately recognise that the kit may be varied so that the contents of two or more containers may be combined. For example, the reagents for PCR and detecting means may be in one container. Furthermore, the first and second containers may be combined to form a single container. The first container may alternatively contain an immobilised antibody specific to the first antibody and the second container may contain the first antibody and be adapted to receive the sample. Additionally, the kit may also contain reagents for the ADA as described in PCT/AU89/00526. All such variations are within the scope of the subject invention.

Accordingly, in one embodiment, the kit will comprise a container having a capturing means for a first DNA containing a target DNA. The capturing means may be an antibody specific to an antigen associated with said first DNA such as an anti-histone antibody or may contain a DNA binding protein such as GCN4 or GST-GCN4. In either event, the antibody or DNA bindingp rotein is immobilised to the surface inside the container. This container is adapted to receive a sample containing a first DNA and is used, after suitable incubations and washings, to amplify and detect the target DNA contained in said first DNA.

One aspect of the present invention is exemplified by the detection of DNA from Plasmodium falciparum in whole blood. Another aspect of the present invention is exemplified by screening for mutant or wild-type alleles of the cystic fibrosis (CF) gene using whole blood. A third aspect of the invention is exemplified by the detection of the human testis specific form of the pyruvate dehydrogenase EI subunit from whole blood or tissue culture. These exemplifications are done with the understanding, however, that the subject method can be used to detect a range of target DNA sequences and is not limited solely to the detection of the genes described herein. Minor modifications may be required when the subject method is applied to the detection of other target DNA sequences or the use of other sources of DNA.

The following description of the immuno-PCR procedure is for the purposes of examplification only and the procedures extend where appropriate to the capture of DNA using DNA binding proteins.

Using anti-histone antibodies in a capture assay in polypropylene microcentrifuge tube, chromosomal DNA can be selectively bound in the presence of a large amount of organic material (eg. blood) and then PCR performed in the same tube. The antibody coated tubes can be stored for at least 1 week, in the cold in PBS containing 0.1% (w/v) azide without significant loss in activity. A minimum of manipulations is required which is an advantage when handling hazardous material. Moreover, this immuno-PCR procedure would seem to be easily adapted for field use, as there are a low number of manipulations and no centrifugation steps are required. During the actual collection of material, no freezing facilities are required and once the chromatin has been captured in the antibody coated tubes, it can be stored dried for at

least 1 week before PCR. Because the volume of sample required is small, 5 µl, it can also be obtained by scratching the surface of the frozen contents of a vial with a pipette tip; thus a precious sample (eg. primary isolates) need not be thawed. Where large numbers of samples need to be processed, immuno-PCR could be performed in microtitre trays.

In the present Examples the anti-malaria histone antibody is effective, when the starting material has a parasitaemia as low as 1%. The antibody used to exemplify the present invention was obtained from mice which had received only 2 injections of P. falciparum histones. It is probable that a more avid antibody obtained from hyperimmunization might be more efficacious, or a monoclonal antibody against histone specific for a particular organism could be used. Alternatively, there are ways of increasing the amount of functional antibody bound to the tube, eg. by coating the tube with protein A or protein G which are polyvalent for the Fc of immunoglobulin (thus, not only might more antibody be bound but all the antibodies would be orientated in the appropriate way) or by using cross-linking agents, eg. carbodimide.

The detection of the CF gene is exemplified in Example 6.

One skilled in the art will readily see the applicability of these techniques to detect any number of different target genes or genetic sequences and the present invention is in no way limited to its exemplified embodiments as described in the following non-limiting Figures and Examples:

In the Figures:

Figure 1 is a graphical representation showing the optimum concentration of immunoglobulin for coating polypropylene tubes. Protein A purified fraction of human anti-histone serum at 0, 5, 10, 30, 90 µg/ml in PBS was added to polypropylene tubes, incubated for 2h at room temperature and washed 3X in PBS. ELISA was performed using peroxidase conjugated sheep anti-human Ig.

Figure 2 is a series of photographs of agarose gel showing the $MgCl_2$ concentration dependence of the amplification of the 316 base pair fragment of the PDH gene and the 193 base pair fragment of the CF gene. Arrows point to the amplified product.

a. is a 1% agarose gel showing $MgCl_2$ concentration dependence. Lane 1 is Drigest III markers; Lane 2 shows $MgCl_2$ concentration of 0.0mM; Lane 3 shows $MgCl_2$ concentration of 1.5mM; Lane 4 shows $MgCl_2$ concentration 2.25mM; Lane 5 shows $MgCl_2$ concentration of 3.0mM; Lane 6 shows $MgCl_2$ concentration of 3.75mM; Lane 7 shows $MgCl_2$ concentration of 4.5mM: Lane 8 shows $MgCl_2$ concentration of 5.25mM; Lane 9 shows $MgCl_2$ concentration of 6.0mM; Lane 10 shows $MgCl_2$ concentration of 7.5mM; Lane 11 shows positive control using Plasmid DNA.

Arrows show amplified product.

b. is a 1% agarose gel showing $MgCl_2$ concentration dependence. Lane 1 shows a BRL 1 Kb ladder; Lane 2 show $MgCl_2$ concentration of 3mM; Lane 3 shows $MgCl_2$ concentration of 3.75mM; Lane 4 shows $MgCl_2$ concentration of 7.5mM; Lane 5 shows $MgCl_2$ concentration of 10.5mM; Lane 6 shows positive control using Plasmid DNA.

Arrows show amplified product.

c is a 1.5% agarose gel showing $MgCl_2$ concentration dependence of the amplification of the 193 base pair fragment of the CF gene. Lane 1 shows a BRL 1Kb Ladder; Lane 2 shows a $MgCl_2$ concentration of 2.5mM and human anti-histone antibody (HHAb) coating concentration of 10µg/ml; Lane 3 shows a $MgCl_2$ concentration of 5.0mM and human anti-histone antibody (HHAb) coating concentration of 10µg/ml; Lane 4 shows a $MgCl_2$ concentration of 2.5mM and murine anti-histone antibody (MHAb) coating concentration of 1:3000; Lane 5 shows $MgCl_2$ concentration of 5.0mM and MHAb coating concentration of 1:3000; Lane 6 shows $MgCl_2$ concentration of 2.5mM and MHAb concentration of 1:25000; Lane 7 shows $MgCl_2$ concentration of 5.0mM and MHAb concentration of 1:25000; Lane 8 shows positive control using genomic DNA.

Arrows show amplified product.

Figure 3 is a photographic representation showing the specificity of immuno-PCR. Polypropylene tubes were coated with human anti-histone (Ab+) or normal Ig (Ab-). Blood infected with Plasmodium falciparum malaria (Ag+) or uninfected blood (Ag-) was added to the tube containing 0.5% (v/v) Triton X-100 in TE. Additional salt was (10X PBS+) or was not (10X PBS-) added. PCR was performed and the products electrophoresed in 1% (w/v) agarose gel containing 0.1 µg/ml ethidium bromide for staining DNA. The far right lane shows the DNA size markers (EcoRI cut spp-1).

Figure 4 is a photographic representation showing the effect of varying the EDTA concentration to immuno-PCR. As for Fig. 2 except that 1, 10 and 100 mM EDTA was used instead of TE, and in one tube mouse anti-P. falciparum histone (M P. falciparum) was used instead of human anti-histone autoantibody.

Figure 5 is a photographic representation showing the sensitivity of immuno-PCR. Using tubes coated with a mouse anti-P. falciparum histone, blood containing 0.04, 0.2 and 1% parasitaemia was used as the source of DNA for immuno-PCR and the products treated as for Fig. 2.

Figure 6 is a photographic representation of agarose gels showing the effect of $MgCl_2$ concentration on the efficiency of amplification of the PDH or CF target sequence using blood and tissue culture cells as the source of DNA, using the DNA binding protein GCN4 as the chromatin capture source.

a. Lane 1 shows a Plasmid DNA (+ve control); Lane 2 shows an $MgCl_2$ concentration of 3mM; Lane 3 shows an $MgCl_2$ concentration of 3.75mM; Lane 4 shows a $MgCl_2$ concentration of 7.5mM; Lane 5 shows a $MgCl_2$ concentration of 10.5mM

b. Lane 1 shows a BRL 1 Kb ladder; Lane 4 shows a $MgCl_2$ concentration of 1.5mM and tube coating with GST-GCN4; Lane 7 shows Plasmid DNA (as a positive control)

c. Lane 1 shows a BRL 1 Kb ladder; Lane 2 shows a 2.5mM Mg^{2+} 1.5×10^6 cells; Lane 3 shows a 2.5mM Mg^{2+} 0.75×10^6 cells; Lane 7 Genomic DNA (as a positive control)

Figure 7 is a photographic representation showing amplified product of the PDH target gene.

Lane 1 shows Genomic DNA as a positive control; Lane 2 shows amplification from 1×10^4 cells; Lane 3 shows amplification from 1×10^5 cells; Lane 4 shows amplification from 1×10^6 cells; Lane 5 shows a BRL 1 Kb ladder

Figure 8 is a graphical representation showing the combined immuno-PCR/ADA on a sample of human blood to detect the CF gene. Conditions are as described in Example 5.

EXAMPLE 1

MATERIALS AND METHODS

Isolation of Histones

1. Plasmodium falciparum histones

A modification of the procedure of Caplan[5] was followed. Aliquots of 4 ml of packed malaria-infected human red blood cells with a parasitaemia of 10% were washed in 0.01M phosphate buffered saline pH 7.5 (PBS) and lysed in 0.5% (v/v) Triton X-100 (Sigma, St.Louis) for 1h at 4°C and nuclei were sedimented at 4000g for 15 min. After washing in 0.5% (v/v) Triton X-100 (4000g for 15 min) the nuclear pellet was suspended in 3 ml of 0.14M NaCl/0.05M $NaHSO_2$ and stirred gently for 15 min. Three washes in the same solution at 4000g for 10 min resulted in a chromatin pellet. Histones were extracted with 1.5 ml of 0.4M NaCl for 20 min with gentle stirring. Two more extractions with 0.75 ml of 0.4M NaCl followed. The addition of 10 volumes of cold acetone to the pooled supernatant precipitated the histones after overnight incubation at -20°C. The histone precipitate was resuspended in 1 ml of 0.3M NaCl.

2. Human histones

The procedure of Baxter et al[6] was followed for the extraction of human histones from the cell line SupT1.

Anti-histone Mouse Antibodies

1. Plasmodium falciparum Experiments

CBA x BALB/c mice were given two intraperitoneal injections (one month apart) of 50µg of histone emulsified in Freund's Incomplete Adjuvant. Serum was obtained 3 weeks after the last injection.

Immunoglobulin was purified on protein A agarose beads.

2. PDH and CF Experiments

Antibodies were raised in bcl-2 transgenic mice (Strasser et al, manuscript in preparation) by two intraperitoneal injections (3 weeks apart) of 50µg human histones. The serum obtained two weeks after the last injection was shown to react against human histones with a titre of 1/25,000.

Human anti-histone antibodies

Human anti-histone antibodies were purified on protein A agarose from serum from a patient treated with hydralazine (such patients often develop autoantibodies to histones). The serum was a gift from Dr S Whittingham, The Walter and Eliza Hall Institute, Melbourne, and was previously shown to react against calf and human histones Western blot analysis.

Oligonucleotides

1. <u>For a testis-specific form of the human pyruvate dehydrogenase EIα subunit</u> (PDH)

The 5' specific primer (P1) has the sequence 5'-TCGCGGTTTCTGTCACCTGT-3' and the 3' specific primer (P2) has the sequence 5'-ATAAAGTCAAACAGATCTCA-3'. The size of the expected DNA fragment is 316 base pairs.

2. For the wild type allele of the cystic fibrosis gene (CF)

The 5' specific primer (CFI) has the sequence 5'-GACTTCACTTCTAATGATGATTAT-3' and the 3' specific primer has the sequence 5'- CTCTTCTAGTTGGCATGCTTTGAT-3'. The size of the expected DNA fragment is 193 base pairs.

3. For the Plasmodium falciparum gene

Oligonucleotides used as primers corresponded to the first and last 30 nucleotides (94-123 and 859-888) of the translated FC27 MSA-2 gene sequence described in Ref[7].

ELISA

This was done as previously described[8].

CHROMATIN CAPTURE

1(a) Using antibodies

Polypropylene microcentrifuge tubes were coated with 50µl of antihistone antibodies using either human anti-histone antibodies (H∝HAb) or normal human IgG at 10µg/ml in PBS or mouse anti-histone antibodies (M∝HAb) at 1/3000 dilution in PBS. Coating was allowed to proceed for 2 hours at room temperature. After three washings in 1 x PBS, 50µl of a nuclear lysis buffer (10mM Tris-HCl/10mM EDTA pH 8.0 + 0.5% Triton x- 100) was added to the tubes followed by 5µl of human heparinized blood. Tubes were mixed gently and after 5 minutes incubation at room

temperature 2.5 µl 5MNaCl was added and the tubes left for 2 hours at room temperature. The contents of the tubes were then discarded and the tubes washed 3 times with PBS. PCR was performed directly in the tubes using the captured chromatin as the source of DNA.

1(b) Using antibodies and proteinase K

Microtitre plates (Nunc-Immuno Module, Maxisorp U16) were coated with 100µl per well of mouse anti-histone antibodies (antibody production previously described) at 1:1000 dilution in 0.1 M Glycine, 0.13M NaCl. The plates were incubated at 37°C for 1 hour and then blocked with a 100µl per well of 0.2% sodium casein in PBS. The plates were incubated for 1 hour at 37°C and then washed 2x with PBS containing 0.2% Tween 20.

Tissue culture cells (SupT1 mammalian cell line) were pelleted and washed 2x in PBS. The cells were resuspended in nuclear lysis buffer (10mM Tris-HCl, 10 mM EDTA pH 8.0 + 0.5% Triton x-100) and the equivalent of 5 x 10^3, 1 x10^4, 1 x 10^5 and 1 x 10^6 cells were added to a coated well in 100 µl of buffer. After a 5 minute incubation 4.8µl of 5 M NaCl was added with mixing. The plate was incubated for 1 hour at 37°C and then washed 6x in PBS containing 0.2% Tween 20. The captured chromosomal DNA was then treated with proteinase K by the addition of 100µl per well of lysis buffer (50 mM KCl, 10 mM Tris - HCl pH 8.4, 0.45% Nonidet P40, 0.45% Tween 20 containing 16 µg per ml proteinase K). The plate was covered with a plate sealer and incubated in a 60°C water bath for 1 hour. The contents of each well were transfered to micro-

centrifuges tubes. Tubes were incubated at 95°C for 10 minutes to inactivate the proteinase K. The volume of each tube was corrected to 100µl with sterile distilled water and a 25µl sample was taken for the PCR.

2. Using DNA binding proteins

Purified GCN4 protein has been purified as a recombinant fusion protein with glutathione S-transferase (GST-GCN4).

Polypropylene microcentrifuge tubes were coated with 50µl of GST-GCN4 at 5µg/ml in PBS. After 2 hour at room temperature the tubes were washed 3x with PBS. The procedure for capture of chromosomal DNA from heparinized blood was as described for the immuno-PCR capture. PCR was performed directly in the tubes as previously described.

For the capture of chromosomal DNA from mammalian tissue culture cells (SupT/1cell line) an alternative nuclear lysis buffer was used. Tissue culture cells (0.75×10^6 and 1.5×10^6 cells) were pelleted and washed 2x in PBS. The cells were resuspended in 50 µl of a lysis buffer containing 50 mM KCl, 10 mM Tris-HCl (pH 8.4), 2.5 mM $MgCl_2$, 0.45% Nonidet P40 and 0.45% Tween 20 and added to the coated tubes. After 5 minutes incubation, 6 µl of 5M NaCl was added with mixing. The tubes were left for 2 hours at room temperature, the contents were then discarded and tubes washed 3x with PBS. A final wash with 50 mM KCl, 10 mM Tris-HCl (pH 8.4) was performed and the PCR conducted directly in the tubes using the capture DNA as the source of DNA.

PCR

Amplification was performed in a 50µl reaction volume containing 10mM Tris-HCl pH 8.4, 50mM KCl, 1.5-7.5mM $MgCl_2$, 200 µM of each deoxynucleotide triphosphate (dATP, dTTP, dCTP, dGTP), 0.2µM of P1 & P2 primers or 0.3µM of CF1 and CF2 primers, and 2.5 units of Taq polymerase. The PCR was overlayed with mineral oil. Amplification was performed in a DNA Thermal Cycler using a predenaturation step at 95°C for 5 minutes, after which the Taq polymerase was added followed by a step-cycle programme of 95°C for 1 minute, 55°C for 1 minute, 72°C for 1 minute for the PDH fragment, 95°C for 1 minute, 50°C for 1 minute and 70°C for 1 minute for the P. falciparum fragment and 95°C for 2 minutes, 40°C for 2 minutes, 65°C for 2 minutes for the CF fragment. All step cycles were performed for 35 cycles. Plasmid DNA or genomic DNA was used as a positive control in each PCR run.

DETECTION OF AMPLIFIED PRODUCT

For each PCR sample 10µl of product was mixed with 2µl of marker dye and electrophoresed on a 1.5% agarose gel. 2µg of a 1 Kilobase DNA ladder (BRL) was run in one lane. The gel was stained in ethidium bromide (diluted to 1µg/ml) and DNA fragments were visualised on a UV box and photographed.

EXAMPLE 2

IMMUNO-PCR

A DNA fragment of the expected size (approximately 310 bp for the PDH gene and 190 bp for the CF gene) was observed in the samples from tubes coated with H HAb's and M HAb's but no visible band was obtained from tubes coated with

normal immunoglobulin. The efficiency of the amplification was dependent on magnesium chloride concentration.

The optimum magnesium concentration for amplification of the PDH gene fragment was 7.5mM and for the CF gene fragment it was 2.5 mM. (Figs 2a, 2b and 2c).

Fig 2a and 2b are 1% (w/v) agarose gels showing the $MgCl_2$ concentration dependence of the amplification of the 316 base pair PDH gene fragment. Arrows indicate amplified product.

Fig 2c is a 1.5% agarose gel showing the $MgCl_2$ concentration dependence of the amplification of the 193 base pair fragment CF gene. An arrow indicates amplified product.

EXAMPLE 3

OPTIMISATION OF IMMUNO-PCR

Optimum concentration of immunoglobulin for coating the polypropylene microcentrifuge tubes.

Aliquots of 50µl of various concentrations of Ig (0, 5, 10, 30 and 90µg/ml PBS) were used to coat polypropylene microcentrifuge tubes for 2h at room temperature. After 3 PBS washes, ELISA was performed using peroxidase conjugated sheep anti-human Ig to determine what the lowest concentration of Ig in the coating solution for optimum binding. This was found to be 10µg/ml (Fig 1) which is similar to the optimal concentrations of protein for coating polystyrene surfaces.

The effect of the buffer constituents on immuno-PCR

The approach was based on the following rationale. Triton X-100 lyses the cell membrane. Divalent cations keep the chromatin insoluble and whole blood contains 2.4mM Ca^{2+} and 1.8 mM Mg^{2+}. Because this would be diluted 1 in 10, ≥ 1 mM EDTA was used in the buffer. EDTA also has the advantage of inhibiting deoxyribonucleases. Disruption of the nuclear envelope would also have to be achieved to allow the antibody access to the chromatin.

Because of these considerations, the following were added to the human anti-histone antibody coated tubes or tubes coasted with normal human Ig: 45µl of 0.5% (v/v) Triton X-100 in 10 mM Tris/1 mM EDTA pH 8.0 and 5µl of human blood at approximately 50% haematocrit and with a 20% parasitaemia of Plasmodium falciparum cultured in vitro. After mixing and standing for 10 min, 5µl of a 10 times PBS solution was added to some tubes. After 2h incubation and three times PBS washes, PCR was done directly in the tubes, using the captured chromatin as the source of DNA. As shown in Fig 3, only the tube which was coated with anti-histone antibody, which contained infected blood and received the extra salt, produced a DNA fragment of the expected 70 base pairs. When normal Ig, normal blood or no salt was used, no visible band was obtained.

If the 10 times PBS was added initially with the blood and Triton X-100 buffer, there was no visible DNA fragment, presumably because the nuclear envelope was not disrupted and hence the chromatin was not accessible. PCR was successful under these conditions, however, if the blood sample had been frozen and thawed, thus exposing some chromatin (data not shown). If 0.5%

deoxycholate was used instead of 0.5% (v/v) Triton X-100, immuno-PCR was unsuccessful presumable because the DNA was released from the histones[9].

For a field study, especially for hazardous material, a minimum of handling is important and fresh samples of blood from needle pricks are often collected. Thus, a buffer was sought that had sufficient salt to waive the 10 times PBS step and yet was able to lyse the nuclear membrane without a freeze/thaw cycle. As shown in Fig 4, although with 10 mM EDTA a PCR product was visible on ethidium bromide staining, 100 mM EDTA was the optimal solution to use. This was somewhat surprising as 100 mM EDTA pH= 8.0 would contain about 200 mM Na and hence be isotonic. Perhaps this excess of chelating agent destabilises nuclei enough (by making divalent cations completely unavailable) to allow chromatin to escape[10]. Also shown in Fig 4 is that either the human autoantibody, or the mouse anti-malaria histone, was effective in immuno-PCR. Subsequently, 0.5% (v/v) Triton X-100 in 100 mM EDTA was used in experiments.

Sensitivity of Immuno-PCR

The results described above used cultured P. falciparum with a parasitaemia ranging from 5 to 20%. To determine how sensitive the immuno-PCR assay was, a P. falciparum culture and a parasitaemia of 20% was diluted in fresh human whole blood to a parasitaemia of 1%, 0.2% and 0.04%. The parasites were mainly in the ring stage of development to mimic the clinical situation. An aliquot of 5µl of this was then added to the antibody-coated tube containing 0.5% (v/v) Triton X-100 and 100 mM EDTA and immuno-PCR was performed as above. When the tubes were coated with mouse anti-malaria histone antibody, a visible DNA fragment is obtained at 1% parasitaemia (Fig

5) but not at 0.2% nor 0.04% parasitaemia. Under the same conditions when human anti-histone autoantibody was used, no visible DNA fragment was obtained at all three concentrations of parasites.

EXAMPLE 4

NON-IMMUNO-PCR USING DNA BINDING PROTEIN

A procedure for the capture of chromosomal DNA was developed utilising the yeast regulatory protein, GCN4[11,12]. GCN4 has been shown to bind a 9 base sequence. Protein dimers of GCN4 bind double-stranded DNA and not single-stranded DNA[13,14]. Although GCN4 has a very high specific affinity for the target sequence of GGATGACTC, experiments described here show that GCN4 also possesses a non-specific affinity for double-stranded DNA sequences, sufficient to capture chromosomal DNA from heparinized blood and mammalian tissue culture cells. Two target sequences, the Human testis-specific form of pyruvate dehydrogenase EI subunit and the wild-type allele of cystic fibrosis were amplified from captured DNA from blood. Captured DNA from tissue culture cells was used to amplify a target sequence from the cystic fibrosis gene.

DNA fragments of expected size (approximately 310 base pairs from the PDH gene and 190 base pairs for the CF gene) were observed. Figs 6a, 6b and 6c show the results.

Fig 6a is a 1.5% (w/v) agarose gel showing the effect of $MgCl_2$ concentration on the efficiency of amplification of the PDH target sequence using blood as the source of DNA.

EXAMPLE 5

A modified protocol for the immuno-PCR method is described which involves the capture of chromosomal DNA from tissue culture cells by antihistone antibodies, followed by digestion of nuclear proteins with proteinase K. It can be argued that digestion of the nuclear proteins will breakdown the nucleosome structure and result in the decondensation of chromatin. This may make target DNA sequences more readily accessible to primers during the polymerase chain reaction. The procedure was performed in a 96 well microtitre plate and required no centrifugation steps. The extracted DNA was added to a tube for amplification. The procedure is rapid and may be used to process many samples at one time.

A target sequence from the Human testis-specific form of pyruvate dehydrogenase E1α subunit was used to exemplify the applicability of this method.

Method

1. Chromatin capture

Amplification was performed in a 50µl reaction volume by mixing a 25µl DNA sample with an equal volume of PCR buffer (10 mM Tris-HCl pH 8.4, 50 mM KCl) containing dNTPs, primers and $MgCl_2$. The PCR was performed as previously described with 2.5 units Taq polymerase being added after the 5 minute 95°C denaturation step. 10µl of the PCR product was electrophoresed and DNA fragments identified.

A DNA fragment of the expected size (approximately 310 base pairs) was observed for the PDH target gene, using 1 x 10^4, 1 x 10^5 and 1 x 10^6 cells. Fig 3 shows the amplified product.

EXAMPLE 6

IMMUNO-PCR/ADA

This example shows the applicability of immuno-PCR to ADA and the combined use of these techniques to identify a target DNA sequence, in this case the wild-type allele of the recently described cystic fibrosis (CF) gene[15].

In accordance with the exemplified protocol of the present invention, the specimen used was whole blood.

There are three possible genotypes in respect of the present deletion in the CF gene in individual subjects:

(i) **Phenotypically Normal**

Where there are two copies of CF wild-type allele in the sample.

(ii) **Phenotypically Normal but Carrier**

Where there is one wild-type and one mutant allele.

(iii) **Phenotypically abnormal - ie. having the disease**

Where there are two copies of the mutant allele.

The protocol involved performing the PCR with three oligo primers (below). Samples of the amplified product were transfered manually to TyrR or GCN4 coated wells. Alternatively, the amplified product can be transfered using coated pins as described in PCT/AU89/00526.

1. **Immuno-PCR**

Mouse anti-human leukocyte histones were purified on protein A agarose beads and used to coat polypropylene microcentrifuge tubes at 10ug/ml (Example 1). After washing three times in PBS (3xPBS), 50ul of 0.5% (v/v) Triton X- 100/10mM EDTA pH8 was added. Five ul of heparinized blood from a healthy adult human whose genotype with respect to the CF gene was unknown was added and mixed. After 5 min, 2.5ul of 5M NaCl was added. After 2 hours, the tubes were washed (3xPBS).

PCR was done in an Innovonics robot arm machine using all 3 oligonucleotides in the one tube:

a) Biotinylated-CGAAATTAAGCACAGTA

b) GGTGTGTAAATATATATTTACACAAAACACCAATGATAT which contains the binding site of tyrR and wild type CF sequence.

c) GGATGACTCAAACACCAATGATAT which contains the binding site of GCN4 and mutant CF sequence.

The oligonucleotides used in this example are different to those used in Example 2 only because they correspond to different regions of the genetic sequence.
The following conditions were used: 35 cycles of 95°C x30", 36°C x 60" and 65°C x 30".

2. ADA

This method was modified from that described in PCT/AU89/00526. Briefly, 50ul of an avidin peroxidase solution (made by adding 2ul of 0.1M ATP, 5ul of 10% (v/v) Tween 20, 4ul of salmon sperm DNA and 10ul of avidin-peroxidase to 1ml of 10% (w/v) skim milk powder/PBS) and 2ul of PCR product were added to microtitre wells coated with either GST-GCN4 or TyrR. After 45 min, wells were washed 3 x with PBS. Peroxidase activity was measured colourimetrically.

3. RESULTS

As shown in Figure 5, the blood specimen give a significant signal only when the well of the ADA has TyrR thus binding the PCR product containing the wild type sequence. Only a background signal was seen in the well which has been coated with GST-GCN4 indicating that the oligo primer containing the GCN4 binding site had not been incorporated to any significant extent in the PCR reaction.

This result was confirmed by agarose gel/ethidium bromide analysis of the products of a PCR reaction using primers which flanked the region of the mutation. The amplified product in this case produced only a single band of the predicted length. Had the mutant allele been present, and the subject therefore a carrier, a second band shorter by three nucleotides - would be seen. This indicates that the mutant allele was not present in the sample. As such, the individual tested is not a carrier of the disease.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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CLAIMS:

1. A method for detecting a target DNA in a sample which method comprises capturing a first DNA containing said target DNA from said sample, amplifying said target DNA using a polymerase chain reaction and then detecting said amplified target DNA.

2. The method according to claim 1 wherein the first DNA is captured by contacting said first DNA with a binding-effective amount of an antibody specific to an antigen associated with said first DNA.

3. The method according to claim 2 wherein the antigen associated with said first DNA is histone and the antibody is an anti-histone antibody.

4. The method according to claim 2 or 3 wherein the antibody is a monoclonal antibody.

5. The method according to claim 1 wherein the first DNA is captured by contacting said first DNA with a binding effective amount of a DNA binding protein capable of associating with said first DNA.

6. The method according to claim 5 wherein the DNA binding protein is specific for double stranded DNA.

7. The method according to claim 6 wherein the DNA binding protein is GCN4.

8. The method according to claim 6 wherein the DNA binding protein is GST-GCN4.

9. The method according to claim 1 wherein the polymerase chain reaction and detection steps are by the amplified DNA assay.

10. A method for detecting a target DNA in a sample which method comprises capturing a first DNA containing said target DNA from said sample by contacting said first DNA with a binding effective amount of an antibody specific to an antigen associated with said first DNA for a time and under conditions sufficient for an antibody-first DNA complex to form, subjecting the captured first DNA to a polymerase chain reaction to amplify said target DNA and then detecting said target DNA.

11. The method according to claim 10 wherein the antigen associated with said first DNA is histone and the antibody is anti-histone antibody.

12. The method according to claim 10 or 11 wherein the antibody is first immobilised on to a solid surface.

13. The method according to claim 12 wherein the solid surface is glass, cellulose, polyacrylamide, nylon, polystyrene, polyvinyl chloride or polypropylene.

14. The method according to claim 13 wherein the solid surface is in the form of a tube, bead, disc or microtitre plate.

15. The method according to claim 12 wherein the antibody is a monoclonal antibody.

16. The method according to claim 10 wherein the amplified DNA is detected by ELISA or spectrophotometric or radioimmunoassay means.

17. The method according to claim 10 wherein the amplification and detection of the target DNA is by the amplified DNA assay.

18. A method for detecting a target DNA in a sample which method comprises capturing a first DNA containing said target DNA from said sample using a DNA binding protein capable of associating with said first DNA, amplifying said target DNA in said captured first DNA using a polymerase chain reaction and then detecting said amplified target DNA.

19. The method according to claim 5 wherein the DNA binding protein is specific for double stranded DNA.

20. The method according to claim 19 wherein the DNA binding protein is GCN4.

21. The method according to claim 19 wherein the DNA binding protein is GST-GCN4.

22. The method according to claim 18 wherein the polymerase chain reaction and detection steps are by the amplified DNA assay.

23. A method for detecting a target DNA in a sample which method comprises capturing on a first solid substrate a first DNA containing said target DNA in said sample and then detecting said target DNA by incorporating a first ligand into said target DNA by a polymerase chain reaction using a set of primers

wherein one of the primers bears the ligand and contacting the so treated DNA with a second solid substrate having a binding reagent for said ligand immobilised thereon and subjecting said captured amplified DNA to a detecting means.

24. The method according to claim 23 wherein the first DNA is captured by contacting said first DNA with a binding effective amount of an antibody specific to an antigen associated with said first DNA.

25. The method according to claim 24 wherein the antigen associated with said first DNA is histone and the antibody is an anti-histone antibody.

26. The method according to claim 24 or 25 wherein the antibody is a monoclonal antibody.

27. The method according to claim 23 wherein the first DNA is captured by contacting said first DNA with a binding effective amount of a DNA binding protein.

28. The method according to claim 27 wherein the DNA binding protein is specific for double stranded DNA.

29. The method according to claim 28 wherein the DNA binding protein is GCN4.

30. The method according to claim 28 wherein the DNA binding protein is GST-GCN4.

31. The method according to claim 23 wherein said detecting means comprises contacting said captured amplified DNA with a detection reagent capable of binding to a second ligand previously incorporated into said amplified DNA by the polymerase chain

reaction using a set of primers wherein one of the primers bears the ligand capable of binding to a detection reagent.

32. A kit for detecting target DNA contained in a first DNA comprising in compartmental form a first container adapted to contain an immobilised antibody specific to an antigen associated with said first DNA and one or more other containers adapted to contain reagents for the PCR and detection of amplified DNA.

33. The kit according to claim 32 wherein the antibody is an anti-histone antibody.

34. A kit for detecting target DNA contained in a first DNA comprising in compartmental form a first container adapted to contain an immobilised DNA and one or more other containers adapted to contain reagents for the PCR and detection of amplified DNA.

35. The kit according to claim 34 wherein the DNA binding protein is GCN4.

36. The kit according to claim 34 wherein the DNA binding protein is GST-GCN4.

37. A method for the selective enrichment and/or isolation of target DNA from specific cells in a mixture of other cells which method comprises contacting a sample containing said cells with an antibody specific to an antigen associated with the genomic DNA containing the target DNA for a time and under conditions sufficient for the antibody to capture the genomic DNA or part thereof, subjecting the DNA so captured to PCR and detecting said

amplified DNA.

38. A method for the selective enrichment and/or isolation of target DNA and/or the identification of certain cell types in a sample which method comprises contacting the cells in said sample with a first antibody specific to an antigen on a desired cell, organism or microorganism type for a time and under conditions to capture said cells, organisms or microorganisms, disrupting said cells, organisms, or microorganisms to release a first DNA containing a target DNA, amplifying the target DNA and then detecting the amplified target DNA.

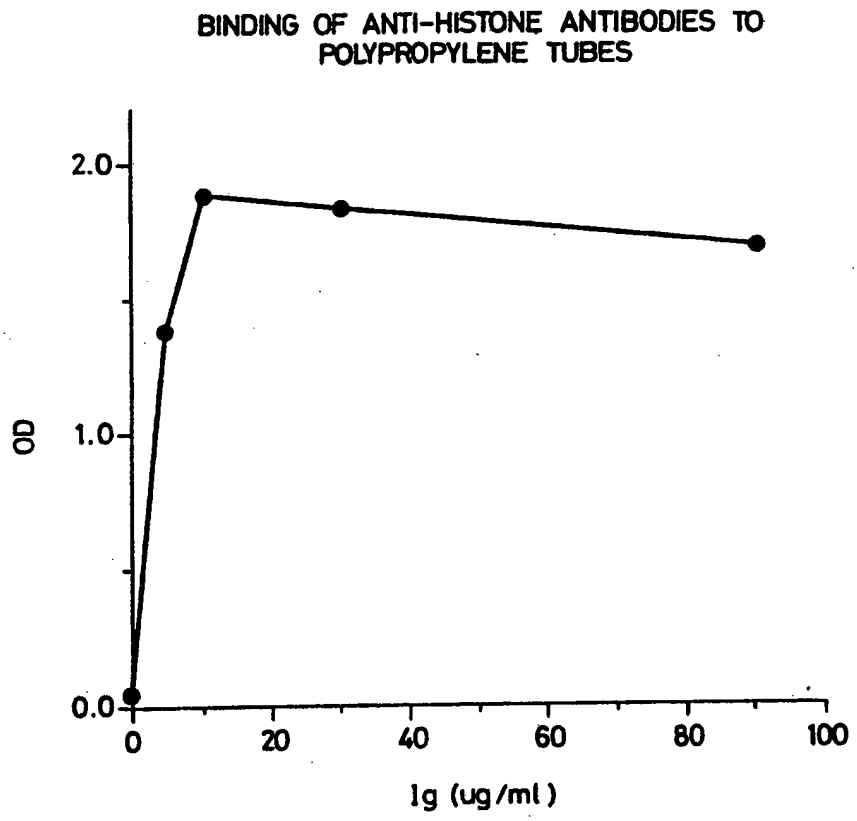
39. The method according to claim 39, further comprising, after the disruption of the cells, organisms or microorganisms, capturing said first DNA.

40. The method according to claim 39 wherein the capture of the first DNA is by a second antibody specific to an antigen associated with said first DNA.

41. The method according to claim 39 wherein the capture of the first DNA is by a DNA binding protein.

42. The method according to claim 40 wherein the first and second antibodies are immobilised on a single solid support.

43. The method according to claim 41 wherein the first antibody and the DNA binding protein are immobilised on a single solid support.


BINDING OF ANTI-HISTONE ANTIBODIES TO POLYPROPYLENE TUBES
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1.0
0.0
OD
0
20
40
60
80
100
lg (ug/ml)

Fig.1.

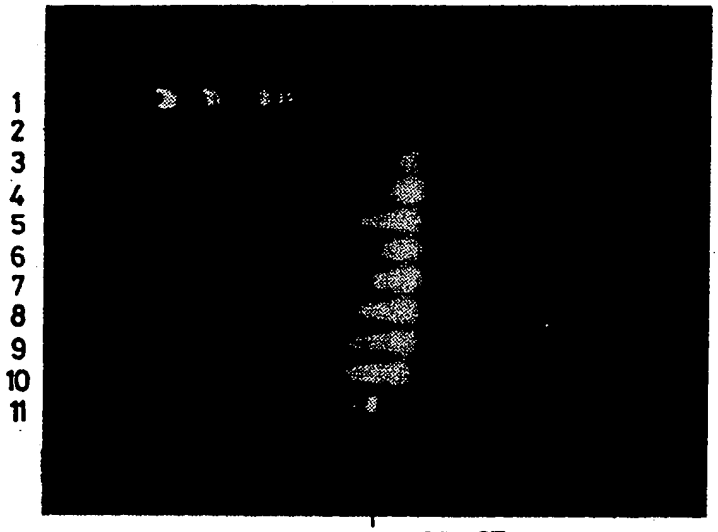

Fig.2(a).

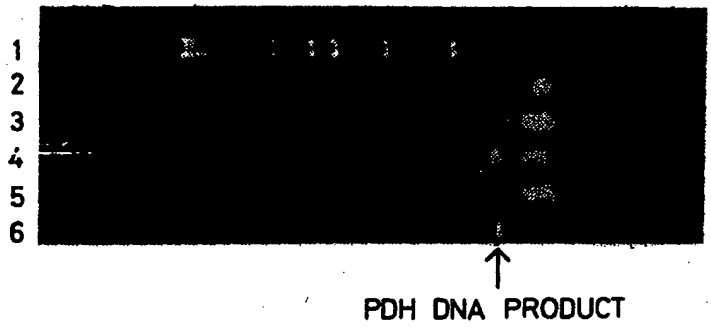

Fig.2(b).

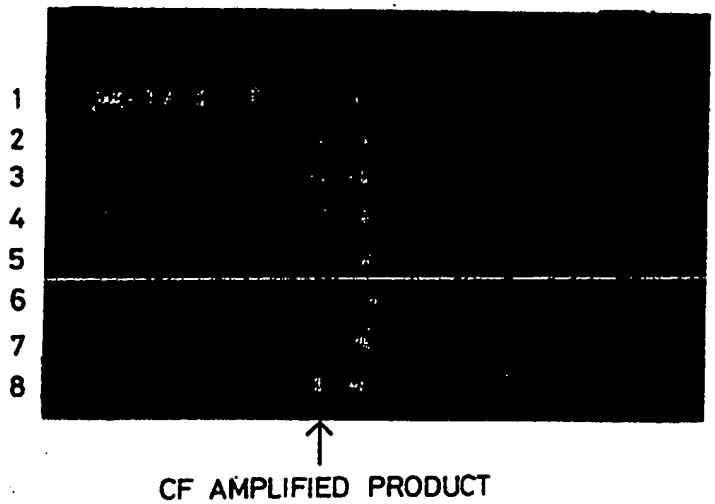

Fig.2(c).

3/6

Ab	−	−	+	+	+	+
Ag	+	+	−	−	+	+
10XPBS	−	+	−	+	−	+

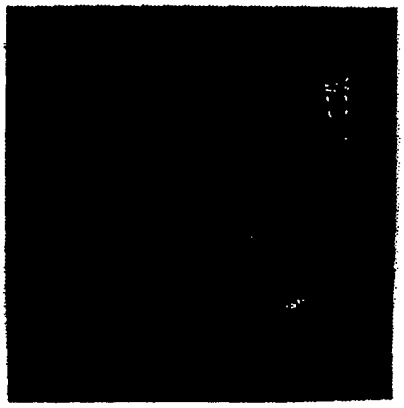

Fig.3.

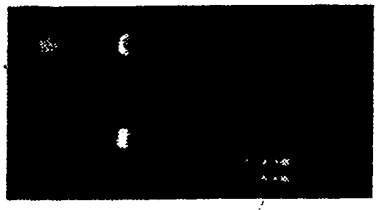

Fig.4.

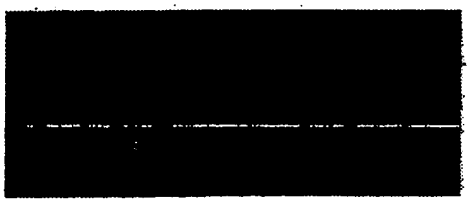

Fig.5.

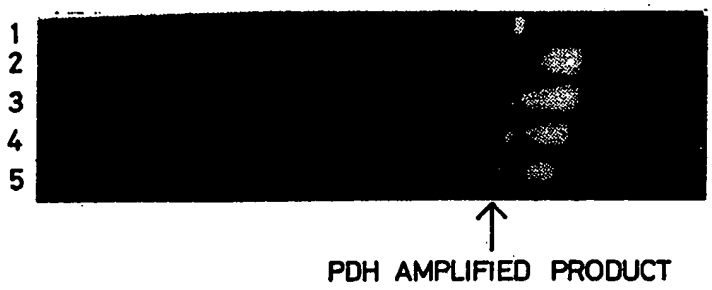

Fig.6(a).

Fig.6(b).

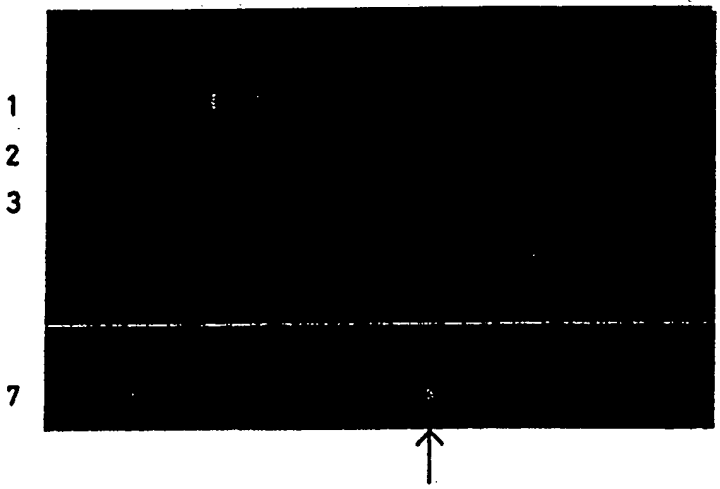

Fig.6(c).

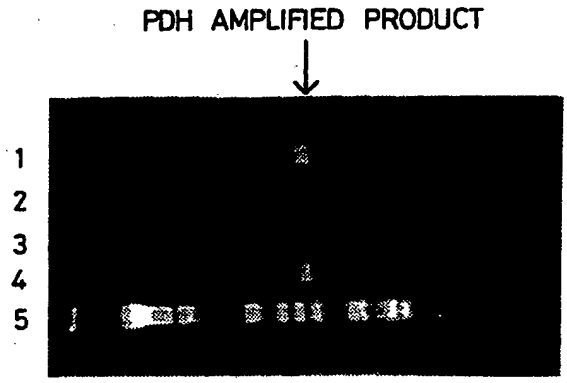

Fig.7.

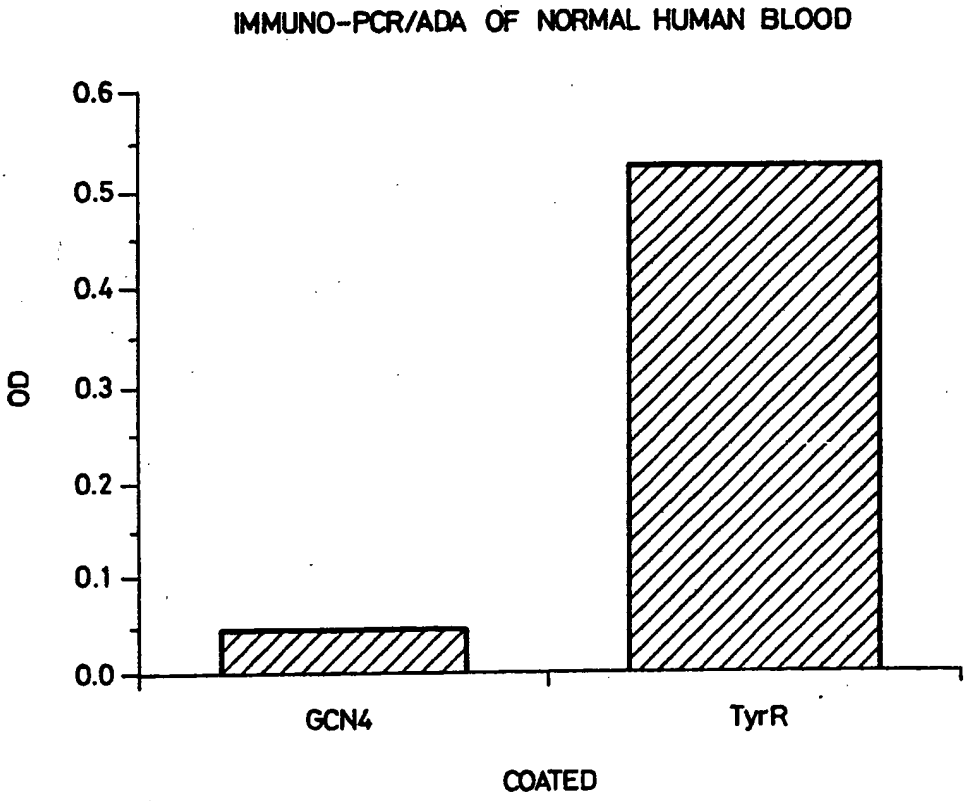
IMMUNO-PCR/ADA OF NORMAL HUMAN BLOOD
0.6
0.5
0.4
0.3
0.2
0.1
0.0
OD
GCN4
TyrR
COATED

Fig.8.

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) 6

According to International Patent Classification (IPC) or to both National Classification and IPC

Int. Cl.[5] C12Q 1/68

II. FIELDS SEARCHED

Minimum Documentation Searched 7

Classification System	Classification Symbols
IPC	WPAT and USPA Derwent Databases : Keywords : "POLYMERASE". "CHAIN", "REACTION", "PCR", "DNA", "ANTIBOD:", "DNA ( ) BINDING ( ) PROTEIN"

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched 8

Chemical Abstracts Keywords : As above
Biotechnology Abstracts Keywords : As above
AU : IPC : As above

III. DOCUMENTS CONSIDERED TO BE RELEVANT 9

Category*	Citation of Document, [11] with indication, where appropriate, of the relevant passages 12	Relevant to Claim No 13
X,Y	AU,A, 27359/88 (AMOCO CORPORATION) 13 July 1989 (13.07.89)	(1-43)
X	AU,A, 33602/89 (CEMU BIOTEKNIK) 5 October 1989 (05.10.89)	(1,23)
X	Zeldis, J.B. et al. Journal of Clinical Investigations, Volume 84, issued November 1989, (The American Society for Clinical Investigations, Inc), "Direct Method for Detecting Small Quantities of Hepatitis B Virus DNA in Serum and Plasma Using the Polymerase Chain Reaction", see pages 1503-1508.	(1,2,10,12-14,23, 24,31,32,37,38)
Y	Kemp, D.J. et al. Proceedings of The National Academy of Science, USA, Volume 86, issued April 1989, "Colorimetric detection of specific DNA segments amplified by polymerase chain reactions", see pages 2423-2427.	(1,5-8,18-22, 34-36)

(continued)

* Special categories of cited documents: 10

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
22 July 1991 (22.07.91)	26 July 91
International Searching Authority	Signature of Authorized Officer
Australian Patent Office	M. BOSS

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

P,Y	AU,A, 46637/89 (AMRAD CORPORATION LIMITED) 14 June 1990 (14.06.90)	(1,5-8,18-22,34-36)
P,X	Panaccio, M. and A. Lew. Nucleic Acids Research, Volume 19(5), issued 1991, (Oxford University Press), "PCR based diagnosis in the presence of 8% (v/v) blood", see page 1151.	(1,5-8,18-22,34-36)
X	Derwent Abstract Accession No. 91-03676, Class L (En) A1 D5 C1, Kemp, D.J. et al. Gene (1990), Volume 94(2), "Simplified colorimetric analysis of polymerase chain reactions: detection of HIV sequences in AIDS patients", see pages 223-228.	(1,5-8,18-22,34-36)
A	AU,A, 55323/86 (CETUS CORPORATION) 2 October 1986 (02.10.86)	
A	AU,A, 55322/86 (CETUS CORPORATION) 2 October 1986 (02.10.86)	

V. [ ] OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE 1

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1.[ ] Claim numbers ..., because they relate to subject matter not required to be searched by this Authority, namely:

2.[ ] Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3.[ ] Claim numbers ..., because they are dependent claims and are not drafted in accordance with the second and third sentences of PCT Rule 6.4 (a):

VI. [ ] OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING 2

This International Searching Authority found multiple inventions in this international application as follows:

1.[ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2.[ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3.[ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

4. [ ] As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

[ ] The additional search fees were accompanied by applicant's protest.

[ ] No protest accompanied the payment of additional search fees.

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON
INTERNATIONAL APPLICATION NO. PCT/AU 91/00131

This Annex lists the known "A" publication level patent family members relating to the patent documents cited in the above-mentioned international search report. The Australian Patent Office is in no way liable for these particulars which are merely given for the purpose of information.

Patent Document Cited in Search Report	Patent Family Members					
AU 27359/88	EP	328829	JP	1211500		
AU 33602/89	EP WO	406296 8909282	NO	904133	SE	8801070
AU 46637/89	CA	2004990	WO	9006374	NO	912169

END OF ANNEX